

REMARKS

Reconsideration and allowance are respectfully requested.

Claims 1-4, 6-8, 10, 12-15, 17-18 and 20-26 are pending. Upon an indication of allowable subject matter, rejoinder is requested of (i) generic or combination/subcombination product claims and (ii) process claims of making/using an allowed product. Claim 20 is amended to facilitate rejoinder.

The amendments are supported by the original disclosure and, thus, no new matter has been added. For example, the limitation of a dependent claim is incorporated in claims 1, 7 and 14. If the Examiner should disagree, however, he is respectfully requested to point out the challenged limitation with particularity in the next Action so support may be cited in response.

The specification was objected to by the Examiner. The hyperlink is rewritten so it is not browser-executable code.

Substitute paper and computer readable forms of the Sequence Listing are being submitted herewith in response to the Examiner's requirement. The paper and computer readable forms of the Sequence Listing do not add new matter, and their contents are the same. It is respectfully submitted that the attached complies with 37 CFR § 1.821 et seq. Otherwise, prompt notice of any defects in the Sequence Listing is earnestly solicited and additional time is requested to comply.

Sequence identifiers are added on pages 5 and 24 of the specification.

Claim 10 was objected to as allegedly informal. It is corrected as suggested by the Examiner. Claim 17 is amended accordingly.

Withdrawal of the objections is requested.

35 U.S.C. 112 – Written Description

Claims 7-10 were rejected under Section 112, first paragraph, because it was alleged that they contain "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed

invention." Applicants traverse because the specification does teach a representative number of species within the claimed genus.

The claims require the use of a packaging signal of SEQ ID NO:1, or fragments thereof, or variants of either. Applicants demonstrate that deletion of SEQ ID NO:1 from the SIV genome renders the virus unable to package RNA. The present specification therefore identifies a signal at the location of SEQ ID NO:1 which is necessary for packaging. The packaging signal identified in this way will clearly have many potential uses. One such use is in the production of vectors that are capable of such packaging. This is what is claimed in the present application. By using a signal contained in SEQ ID NO:1, which is required for packaging, a vector can be produced which is capable of being packaged by SIV; such a vector may be used in combination with a packaging-defective vector to deliver heterologous protein to cells. The nucleotide sequence from a claimed vector could thus be packaged within an SIV viral particle and targeted to cells that are infectable with SIV. See, e.g., pages 7-8 of the specification.

The present claims are therefore directed to vectors which comprise a packaging signal of SEQ ID NO:1, or fragments thereof, or variants of either. This sequence is essential for packaging to occur, and is therefore the minimum sequence required for packaging in such a vector. It identifies the region which is roughly between the primer binding site and the 5' major splice donor as necessary for packaging. See page 7, lines 16-18 and 28-32, and page 8, lines 25-28, of the specification. Not only is the 85 bases of SEQ ID NO:1 taught by Applicants, but also fragments thereof (including nucleotides 53 to 85 of SEQ ID NO:1) and variants (including SIV polymorphisms and mutations in the DIS structure such as those shown in Fig. 3). See page 8, lines 1-12, and page 9, lines 3-21, of the specification.

Clearly, other SIV sequences could also be included in such a vector. As taught on page 13, lines 10-13, of the specification, additional sequences may also be included from the region downstream of the 5' splice donor site up to the gag initiation codon, such as those of SEQ ID NO:2. Further sequences may also be included, depending on the purpose intended for a particular vector. As taught on page 13, lines 24-27, and page 13, line 30, to page 14, line 3, of the specification, the first 500 bp of the SIV gag

sequence can be included in order to enhance packaging efficiency. Similarly, the U3, R and U5 sequences of the LTRs, the packaging sequences and sequences flanking the LTRs may be included in order to allow the SIV sequence to be packaged, reverse-transcribed, integrated into the host genome, and expressed in target cells.

It can thus be seen that the exact sequences present in a vector of the invention may vary, and could be selected by the skilled person on the basis of the teaching in the application, together with their knowledge of this field, to suit the particular purpose of the selected vector. The vector, however, must include a sequence as now claimed, because this forms an essential part of the packaging signal. In contrast to allegations made in the Action, the packaging signal is not limited to SEQ ID NO:1 and Applicants teach a representative number of species within the genus of possible packaging signals. See above citations and page 9, line 29, to page 10, line 2, of the specification.

Withdrawal of the written description rejection made under Section 112, first paragraph, is requested because the specification conveys to a person skilled in the art that Applicants were in possession of the claimed invention as of the filing date.

35 U.S.C. 102 – Novelty

Claims 7-10 were rejected under Section 102(b) as allegedly anticipated by Rizvi & Panganiban (J. Virol. 67:2681-2688, 1993) (hereinafter "Rizvi"). Applicants traverse.

The present invention resulted from Applicants identifying a packaging signal in the SIV genome. As demonstrated in the present application, this sequence is essential for packaging in SIV. The sequence initially identified by Applicants is SEQ ID NO:1. This sequence derives from the region between the primer binding site and the 5' major splice donor site in SIV. In particular, this sequence includes the DIS structure as shown in Fig. 3a.

Rizvi describes the encapsidation of SIV RNA by HIV-1 particles. It suggests that the 5' leader region of retroviral RNA functions as the primary *cis*-acting region necessary for packaging (page 2684, column 1, final paragraph). In particular, the terminal 422 nucleotides of SIV RNA are identified as facilitating encapsidation. This includes

only 92 nucleotides downstream of the PBS and, therefore, does not include the sequence of SEQ ID NO:1.

As taught by Applicants in the paragraph bridging pages 3-4 of the specification, although Rizvi suggests a general location of the packaging signal in SIV, a considerable stretch of the SIV sequence is used by Rizvi, and no attempt was made by Rizvi to define the sequences that would be necessary or sufficient for packaging. Furthermore, the region described by Rizvi includes numerous *cis*-acting sequences, including the primer binding site (PBS), splice donor, and the complete 5' leader region. It is unclear whether Rizvi is suggesting that one of these sequences, a combination of two or more of them, or indeed the entire region used, is supposed to be responsible for packaging in SIV. The results reported by Rizvi therefore do not define sequences that are necessary or sufficient for SIV packaging to an extent that they might actually be useful for the development of SIV as a vector, for example, for the delivery of heterologous proteins to a target cell.

Furthermore, the results reported in Rizvi are based on the concept of using packaging by a second virus in order to locate packaging sequences. Rizvi thus uses HIV-1 vectors to package vectors which comprise hypothetical SIV packaging regions.

The approach in Rizvi relies on the assumptions that (a) the selected HIV-1 vectors are actually capable of packaging SIV and (b) the signals required for the HIV-1 vectors to achieve such packaging of SIV are the same signals that would be used for SIV-self packaging. In fact, this is not the case.

It is not safe to assume that one type of lentivirus is capable of packaging the genome of a different lentivirus. For example, HIV-1 and HIV-2 show a non-reciprocal packaging relationship. Attached is a copy of Kaye & Lever (J. Virol. 72:5877-5885, 1998) for the Examiner's consideration. This document demonstrates that HIV-1 is able to package both HIV-1 and HIV-2 vector RNA, but HIV-2 is unable to package HIV-1 vector RNA. It is thus clear that the ability of one virus to package the genome of a second virus does not imply that the same sequences are the relevant ones for packaging the viral genome using its own proteins. Neither does the absence of

packaging of one virus by another mean that sequences included in the RNA are not relevant for packaging when this occurs in the context of the virus's own proteins.

The results reported in Rizvi thus fail to teach the location of the SIV packaging signal. Furthermore, because these results are based on the use of HIV-1 to packaging the SIV vector, they cannot be used to draw conclusions about which regions of the SIV genome are required or sufficient for viral packaging by SIV proteins.

Claims 7-10 were rejected under Section 102(e) as allegedly anticipated by Lauermann (U.S. Patent 6,635,472) (hereinafter "Lauermann"). Applicants traverse.

Lauermann describes in very general terms some retroviral vectors which may comprise heterologous genes. But although SIV is listed as one possible retroviral source, there is no specific teaching in this document of SIV vectors. The working examples refer only to MLTV and HIV-1 vectors. In fact, the only mention of packaging signals by Lauermann is a brief sentence at column 11, lines 3-4. This provides no indication of where packaging signals might be located, or how the reader could go about determining the location of a packaging signal for a selected virus (e.g., SIV).

Based on Lauermann, therefore, there would be no particular reason to produce vectors based on SIV. Even if SIV was selected as a vector, the artisan is given no help in determining what sequences from SIV should be included in such a vector in order to allow packaging. The artisane would have no reason to produce a vector as claimed which includes, for example, a packaging signal comprising SEQ ID NO:1, or a fragment thereof, or a variant of either.

Withdrawal of the Section 102 rejections is requested because all limitations of the claimed invention are not disclosed by the cited references.

Conclusion

Having fully responded to all of the pending objections and rejections contained in this Office Action, Applicants submit that the claims are in condition for allowance and


LEVER et al. – Appln. No. 10/009,171

earnestly solicit an early Notice to that effect. The Examiner is invited to contact the undersigned if any further information is required.

Respectfully submitted,

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Nonreciprocal Packaging of Human Immunodeficiency Virus Type 1 and Type 2 RNA: a Possible Role for the p2 Domain of Gag in RNA Encapsidation

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The ability of human immunodeficiency virus types 1 (HIV-1) and 2 (HIV-2) to cross-package each other's RNA was investigated by cotransfecting helper virus constructs with vectors derived from both viruses from which the *gag* and *pol* sequences had been removed. HIV-1 was able to package both HIV-1 and HIV-2 vector RNA. The unspliced HIV-1 vector RNA was packaged preferentially over spliced RNA; however, unspliced and spliced HIV-2 vector RNA were packaged in proportion to their cytoplasmic concentrations. The HIV-2 helper virus was unable to package the HIV-1 vector RNA, indicating a nonreciprocal RNA packaging relationship between these two lentiviruses. Chimeric proviruses based on HIV-2 were constructed to identify the regions of the HIV-1 Gag protein conferring RNA-packaging specificity for the HIV-1 packaging signal. Two chimeric viruses were constructed in which domains within the HIV-2 *gag* gene were replaced by the corresponding domains in HIV-1, and the ability of the chimeric proviruses to encapsidate an HIV-1-based vector was studied. Wild-type HIV-2 was unable to package the HIV-1-based vector; however, replacement of the HIV-2 nucleocapsid by that of HIV-1 generated a virus with normal protein processing which could package the HIV-1-based vector. The chimeric viruses retained the ability to package HIV-2 genomic RNA, providing further evidence for a lack of reciprocity in RNA-packaging ability between the HIV-1 and HIV-2 nucleocapsid proteins. Inclusion of the p2 domain of HIV-1 Gag in the chimera significantly enhanced packaging.

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) both cause AIDS. Both are members of the lentivirus subfamily of retroviruses, and they have a similar genomic organization with virtually identical open reading frames. At the nucleotide and amino acid levels, there is limited homology (21), with HIV-2 being more closely related to simian immunodeficiency virus than it is to HIV-1.

An essential step in the retroviral life cycle is encapsidation of the genomic RNA. This process is highly specific and results in the selection of the unspliced viral mRNA for packaging into progeny virions against a high background of cellular mRNAs and subgenomic viral RNAs. The viral genomic RNA is reported to represent approximately 1% of the mRNA in the cytoplasm of an infected cell yet constitutes the vast majority of the mRNA in the virion. The basis for the specificity of RNA packaging has two components: (i) the *cis*-acting RNA packaging signals, known as Ψ (PSI) or E (encapsidation) signals, and (ii) the *trans*-acting factors, namely, the Gag polyprotein, which specifically binds and sequesters the genomic viral RNA for encapsidation (24).

The *cis*-acting sequences required for encapsidation of HIV-1 RNA have been mapped by deletion and mutational analysis (1, 10, 23, 29). Initial studies indicated that the region between the major splice donor and the start of *gag* were important for encapsidation; however, subsequent studies have suggested that other sequences also play an important role (6, 7, 11, 25, 26, 31, 41). Of these regions, a stem-loop upstream of

the major splice donor which contains the putative dimerization initiation signal and a stem-loop at the 5' end of the *gag* gene have been shown to be important for Gag protein binding *in vitro* (6, 7, 11, 43) and RNA packaging *in vivo* (31, 33). The sequences required for encapsidation of HIV-2 RNA have been less intensively studied (19, 35). Deletion analyses have shown that sequences upstream of the major splice donor have a more marked effect on encapsidation of HIV-2 RNA than do sequences between the major splice donor and the start of *gag* (35).

Mutational analyses of the nucleocapsid (NC) domain of HIV-1 Gag have demonstrated the importance of the NC domain for encapsidation of viral genomic RNA (12, 14, 20, 45). The NC domain lies toward the C-terminal end of the Gag polyprotein. The NC proteins of all retroviruses, with the exception of spumaviruses, contain one or two copies of the sequence Cys-X₂-Cys-X₄-His-X₄-Cys, termed Cys-His motifs, which chelate zinc ions (36). NC proteins are highly basic, often containing contiguous doublets or triplets of basic residues flanking the Cys-His motifs. Mutations disrupting the zinc fingers or the flanking basic residues result in defects in viral RNA packaging. *In vitro* RNA-protein binding studies have shown that the Gag polyprotein and the NC domain bind specifically to RNA containing the HIV-1 packaging sequences (6, 7, 11, 12, 43).

The role of the NC domain of HIV-1 in determining RNA packaging specificity has been investigated by using chimeric constructs between HIV-1 and Moloney murine leukemia virus (M-MuLV) (8, 47). The presence of the HIV-1 NC domain in the context of M-MuLV Gag conferred on the M-MuLV chimera an enhanced ability to specifically package HIV-1 genomic RNA. The present study was designed to further

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TABLE 1. Oligonucleotide primers used in sticky feet-directed mutagenesis

Primer	Mutant	Sequence
A1	pSVRM1	5'-AGATTAATGGCTGAAGCAATGAGCCAAGTA-3'
A2	pSVRM2	5'-TTCGCAGCAGCCAGAGAGGCAATTTAGGAAC-3'
B	pSVRM1 and pSVRM2	5'-TAAAAAACACGCTGTCTCTCAGTACA-3'
SFA1	pSVRM1	5'-GGTGGGCCAGGCCAGAAAGCTAGATTATGGCTGAAGCAATGAGCCCA-3'
SFA2	pSVRM2	5'-GGACCTGCCCCATCCCATTCGCAGCAGCCAGAGAGGCAATTTAGG-3'
SFB	pSVRM1 and pSVRM2	5'-CTTTCCCCAAGGGCCCCAGTCCTAAAAAACGACCTGTCTCTCAGTACA-3'

investigate the role of the NC domain of Gag in this process. We constructed vectors based on HIV-1 and HIV-2 so that we could observe cross-packaging by both helper viruses and chimeric constructs of HIV-1 and HIV-2 in which domains within the Gag polypeptide, including NC, of HIV-1 were introduced into HIV-2, and investigated the effect of the domain swaps on RNA encapsidation.

MATERIALS AND METHODS

Plasmid construction. pSVC21 is an infectious proviral clone of the HTLV-III_B isolate that was originally derived from a plasmid (HXBc2) (17) containing a simian virus 40 origin of replication. The HIV-1 packaging construct, pBCCX-CSF, and HIV-1-based vector, HVPΔEC, have been described previously (22, 41). pSVR is an infectious proviral clone of HIV-2 ROD containing a simian virus 40 origin of replication (35). Restriction sites, where given, refer to positions in the retroviral genome (Los Alamos database numbering, where position 1 is the first base of the 5' long terminal repeat for HIV-1 and the first nucleotide in the viral RNA for HIV-2). pSVRΔX was created by introducing an *Xba*I site at nucleotide 553 by oligonucleotide-directed mutagenesis (28) with the mutagenic oligonucleotide 5' CGGAGTTTCTAGAGCCCATCTCC 3'. The sequences between *Xba*I sites at 553 and 5067 were deleted, removing *gag* and *pol* coding sequences. pSVRΔAX was created by deleting the sequences between *Acl*I (position 912) and *Xba*I (position 5067).

The chimeric proviral constructs, pSVRM1 and pSVRM2, were made as follows. An *Aat*II-*Xho*I (nucleotide [nt] 2032) fragment containing the HIV-2 5' long terminal repeat, the untranslated region, and *gag* was cloned from pSVR into pGEM7Zi+ (Promega), creating pGRAXS. Chimeric constructs, pSVRM1 and pSVRM2, were generated by a modification of the "sticky-feet directed mutagenesis" method (9). The PCR primers used for the mutagenesis are shown in Table 1. Primers A1 and B were used to generate a PCR fragment containing the HIV-1 HXBc2 p2 and NC domain with 15 bp of HIV-2 sequence at the 5' and 3' ends. A second PCR was performed on the purified PCR product with primers SFA1 and SFB to generate a PCR product with 30 bp of HIV-2 sequence at each end. Primers A2 and B, followed by SFA2 and SFB, were used similarly to generate a PCR product containing the HIV-1 HXBc2 NC domain flanked by 30 bp of HIV-2 sequence. The reverse primer, SFB, was biotinylated at the 5' end to facilitate the removal of this strand with streptavidin-coated magnetic beads (Dyna) as specified by the manufacturer. The single-stranded PCR products were used to mutate the HIV-2 sequence in pGRAXS (28). The chimeric *gag* sequences were cloned back into pSVR by the using *Aat*II and *Xho*I restriction sites. All constructs were subjected to nucleotide sequencing to confirm that the mutated sequences were as expected. All HIV-2 based plasmids were grown in TOP10F⁺ (Invitrogen) at 30°C to minimize recombination. All other plasmids were grown in DH5α.

Plasmids used as templates for the production of riboprobes were constructed as follows. Plasmid KSIIΨCS, used to detect HIV-1 RNA, has been described previously (24). Plasmid KS1SB, used to detect HIV-1 RNA, was created by amplification of HIV-1 sequences from positions 403 to 909 with the primers 5' TAATGGATCCAGTGGCGAGCCCTCAGATCCTGCAT 3' and 5' CTTAGTCGACGCTCCCTGCTTGGCCATACTATATG 3'. The PCR product was then cloned into the *Sal*I and *Bam*HI sites of Bluescript KSII (Stratagene). Plasmid KSIIΨ2KE, used to detect HIV-2 RNA, was constructed by cloning the *Ehe*I (nt 306)-*Kpn*I (nt 751) fragment from pSVR into the *Pst*I and *Kpn*I sites of Bluescript KSII (Stratagene). Plasmid KS2ES, used to detect HIV-2 RNA, was created by amplification of HIV-2 sequences from 4915 to 5284 with the primers 5' CATGGAATTCAGGGAGGATGGAGAAATGG 3' and 5' CTTATAGTCGACTCGGGGATAATTGCACGAGG 3'. The PCR product was then cloned into the *Eco*RI and *Sal*I sites of Bluescript KSII (Stratagene).

Cell culture and transfections. COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Transient transfection of COS-1 cells was performed by the DEAE-dextran method (46). Cells and supernatants were harvested 48 to 72 h later. Viral particle production was measured by the reverse transcriptase assay (40).

Protein analysis. COS-1 cells were metabolically labelled with [³⁵S]methionine (>1,000 Ci/mmol) from 44 to 48 h after transfection. Labelled cells were lysed in radioimmunoprecipitation assay buffer (140 mM NaCl, 8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate [SDS]). Virions released into the supernatant were pelleted by centrifugation for 15 min at 4°C and 80,000 rpm in a Beckman TLA-100 rotor. Pelleted virions were lysed in RIPA buffer, and cell and virion lysates were immunoprecipitated with serum from a panel of HIV-2-infected individuals (Medical Research Council AIDS reagent project) before being analyzed in 5 to 20% acrylamide-SDS gradient gels (Bio-Rad).

RNA isolation. Cytoplasmic RNA was obtained by rapid lysis at 4°C in Nonidet P-40 buffer (50 mM Tris-Cl [pH 8.0], 100 mM NaCl, 5 mM MgCl₂, 0.5% [vol/vol] Nonidet P-40). Cell debris and nuclei were removed by a 2-min centrifugation step in a microcentrifuge. The supernatant was adjusted to 0.2% SDS and 125 μg of proteinase K per ml, incubated at 37°C for 15 min, and extracted twice with acid-buffered phenol-chloroform (pH 4.7) and once with chloroform. Nucleic acids were collected by ethanol precipitation, and RNA was stored at -80°C. For RNA extraction from virions, particles released from the cell culture supernatant were pelleted by polyethylene glycol precipitation by the addition of 0.5 volume of 30% polyethylene glycol 8000 in 0.4 M NaCl for 16 h at 4°C. The precipitate was collected by centrifugation at 2,000 rpm in an MSE 43124-129 rotor at 4°C for 45 min and resuspended in 0.5 ml of TNE (10 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA [pH 7.5]). This material was layered over an equal volume of TNE containing 20% sucrose and centrifuged at 98,000 × g for 2 h at 4°C. Virus particles were lysed in proteinase K buffer (50 mM Tris-Cl [pH 7.5], 100 mM NaCl, 10 mM EDTA, 1% SDS, 100 μg of proteinase K per ml, 100 μg of tRNA per ml) for 30 min at 37°C. After two extractions with acid-buffered phenol-chloroform and one extraction with chloroform, the RNA was precipitated with ethanol and stored at -80°C. The isolated RNA was resuspended in 100 μl of a buffer containing 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 10 mM MgCl₂, 1 mM dithiothreitol, 5 U of RNase-free DNase 1 (Promega), and 4 U of RNase inhibitor (Promega) and incubated at 37°C for 15 min. The reaction was stopped by the addition of 25 μl of a solution containing 50 mM EDTA, 1.5 M sodium acetate, and 1% SDS, and the samples were extracted once with acid-buffered phenol-chloroform and once with chloroform. The RNA was precipitated with ethanol.

RNase protection assay. ³²P-labelled riboprobes were synthesized by in vitro transcription of linearized plasmids, KSIIΨCS or KS1SB (HIV-1-specific probes, nt 313 to 830 and 403 to 909, respectively) or KSIIΨ2KE or KS2ES (HIV-2-specific probes, nt 306 to 751 and 4915 to 5284, respectively), with T3 RNA polymerase (Promega). The riboprobes were purified from 5% polyacrylamide-8 M urea gels before being used in RNase protection assays.

Reagents for RNase protection assays were obtained from a commercially available kit (Ambion, Austin, Tex.). Cytoplasmic RNA or RNA extracted from pelleted particles representing one-third of the transfected cells was incubated with 2 × 10⁵ cpm of ³²P-labelled probe in 10 μl of hybridization buffer (Ambion) for 10 min at 68°C. Unhybridized regions of the probe were then degraded by the addition of 0.5 U of RNase A and 20 U of RNase T₁ in 100 μl of RNase digestion buffer (Ambion). Protected fragments were precipitated in ethanol, resuspended in RNA loading buffer, and separated on 5% polyacrylamide-8 M urea gels. For size determination, ³²P-labelled RNA markers synthesized with the RNA Century Marker template set (Ambion) were run in parallel. The gels were subjected to autoradiography, and the relative RNA levels were quantified by densitometry with National Institutes of Health Image software.

RESULTS

Nonreciprocal RNA packaging of HIV-1 and HIV-2. To investigate the ability of HIV-1 and HIV-2 helper viruses to package HIV-1 and HIV-2 RNA, vectors were constructed in which *gag* and *pol* sequences were deleted. This allowed us to determine the ability of the helper viruses to package the vector RNA in *trans*. The vector and helper virus constructs are shown in Fig. 1 and described in Materials and Methods. The HIV-1-based vector, HVPΔEC, has previously been shown to

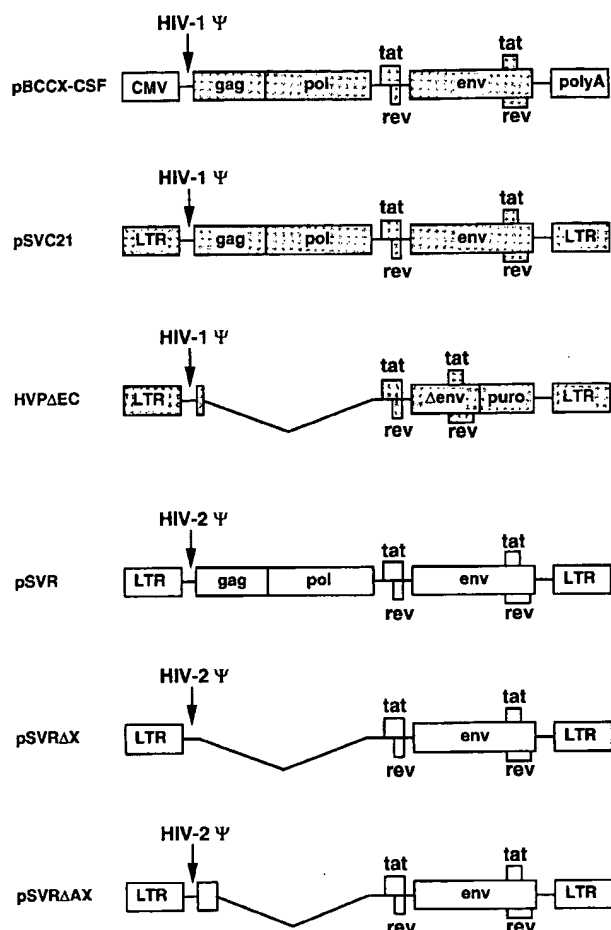


FIG. 1. Schematic representation of helper virus and vector constructs. HIV-1 sequences are shaded. pBCCX-CSF is an HIV-1 particle producer which uses the human cytomegalovirus (CMV) immediate-early promoter to drive the expression of HIV-1 genes. pSVC21 is an HIV-1 proviral construct. HVP Δ EC is an HIV-1-based vector with the sequences between *Cla*I (nt 830) and *Bal*I (nt 2689) deleted and a puromycin resistance gene (*puro*) inserted in the *nef* position. pSVR is an HIV-2 proviral construct. pSVR Δ X was derived from pSVR by deletion of sequences between an introduced *Xba*I site at nt 553 and *Xba*I at nt 5067. pSVR Δ AX was derived from pSVR by deletion of sequences between *Acc*I (nt 912) and *Xba*I (nt 5067). polyA, polyadenylation sequences; Ψ , encapsidation sequences; LTR, long terminal repeat.

be packageable by HIV-1 (41). The HIV-2-based vector, pSVR Δ X, was constructed similarly by deleting *gag* and *pol* sequences, leaving the 5' untranslated region intact (see Materials and Methods for details). HVP Δ EC and pSVR Δ X were cotransfected into COS-1 cells with either HIV-1 or HIV-2 helper virus constructs (pBCCX-CSF or pSVR respectively) and the ability of the vector RNA to be packaged by the helper viruses was analyzed by an RNase protection assay. The results are shown in Fig. 2. As expected, the HIV-1 vector, HVP Δ EC, was packaged by the HIV-1 helper virus (Fig. 2B, lane 7). Full-length vector RNA was packaged preferentially compared to spliced RNA. The HIV-2 helper virus failed to encapsidate the HIV-1 vector. We were unable to detect HIV-1 vector RNA in virions released from cells cotransfected with HIV-2 helper virus (lane 8) despite good levels of expression of vector RNA in the cytoplasm of these cells (lane 4). In contrast, the HIV-2 vector, pSVR Δ X, was not packaged by the parental HIV-2 helper virus (Fig. 2C, lane 7) but was packaged by the

HIV-1 helper virus (lane 8). We were surprised at the failure of the HIV-2 helper virus to package the HIV-2 vector RNA, because only *gag* and *pol* sequences had been removed from the vector and the 5' untranslated region, containing sequences previously shown to be important for packaging (19, 35), was intact. Full-length helper virus RNA was packaged efficiently and preferentially compared to spliced RNA (Fig. 2C, lane 7). The HIV-2 vector was packaged by the HIV-1 helper virus (lane 8); however, the specificity for full-length RNA was lost: the ratio of full-length to spliced RNA in the virions reflected the ratio of unspliced to spliced RNA present in the cytoplasm of the transfected cells. Thus, the HIV-1 helper virus was able to package both HIV-1 and HIV-2 RNAs, although it did not discriminate between unspliced and spliced HIV-2 RNAs.

Construction of chimeric proviruses. The NC domain of the Gag polypeptide has been strongly implicated in RNA recognition. The nucleocapsid domains of HIV-1 and HIV-2 are fairly well conserved, with about 60% amino acid identity. In contrast, the 5' untranslated regions of the viruses have little sequence homology and there are no similar structural motifs (4). We therefore designed two chimeras: the first contained the p2 and NC domains of HIV-1 in place of the corresponding domains in HIV-2, and the second contained only the NC domain of HIV-1 in place of the NC domain of HIV-2. The constructs are shown in Fig. 3. Sticky feet-directed mutagenesis was used to swap the domains precisely (see Materials and Methods for details). We aimed to retain consensus protease cleavage sites at the junctions between the HIV-1 and HIV-2 sequences (Fig. 3B), although we have previously shown that cleavage of the Gag polypeptide is not a prerequisite for HIV-1 RNA recognition and encapsidation (24).

The protein expression, processing, and particle release from the chimeric proviruses was compared to those of the parental HIV-2 provirus. COS-1 cells were transfected with wild-type or chimeric proviral constructs, and cellular and virion proteins were analyzed by an immunoprecipitation assay (Fig. 4). Both chimeric constructs expressed similar levels of viral proteins to those of the parental HIV-2 provirus. Viral antigen was released into the supernatant in pelletable virus particles. The chimeras produced similar levels of reverse transcriptase activity to those of the wild-type virus (data not shown). Thus, protein expression and particle release had not been affected by the domain swaps.

RNA packaging by the chimeric proviruses. The ability of the chimeras to package HIV-1 RNA was assessed by cotransfection of each with HVP Δ EC followed by an RNase protection assay. As expected, the HIV-1 vector was packaged by the HIV-1 helper virus (Fig. 5B, lane 9) and not by the HIV-2 helper virus (lane 10). The HIV-1 vector RNA was packaged by both chimeras, but at a reduced level compared to wild-type HIV-1 (compare lanes 11 and 12 to lane 9). pSVRM1 reproducibly packaged the HIV-1 vector better than pSVRM2 did, suggesting a role for the HIV-1 p2 domain in enhancing packaging. We constructed a second HIV-2 vector in which the sequences between *Acc*I (nt 912) and *Xba*I (nt 5067) were deleted (Fig. 1). The ability of this vector to be packaged by wild-type HIV-1 and HIV-2 and by the chimeras was assessed by cotransfection followed by an RNase protection assay. The HIV-2 vector, pSVR Δ AX, was packaged by the parental HIV-2 helper virus (Fig. 5C, lane 8). It was also packaged by wild-type HIV-1 and by the chimera pSVRM1 (lanes 9 and 10, respectively), indicating that the combined HIV-1 p2 and NC domains of Gag are able to encapsidate an RNA containing the appropriate HIV-2-packaging signals.

The ability of the chimeras to package their own genomic

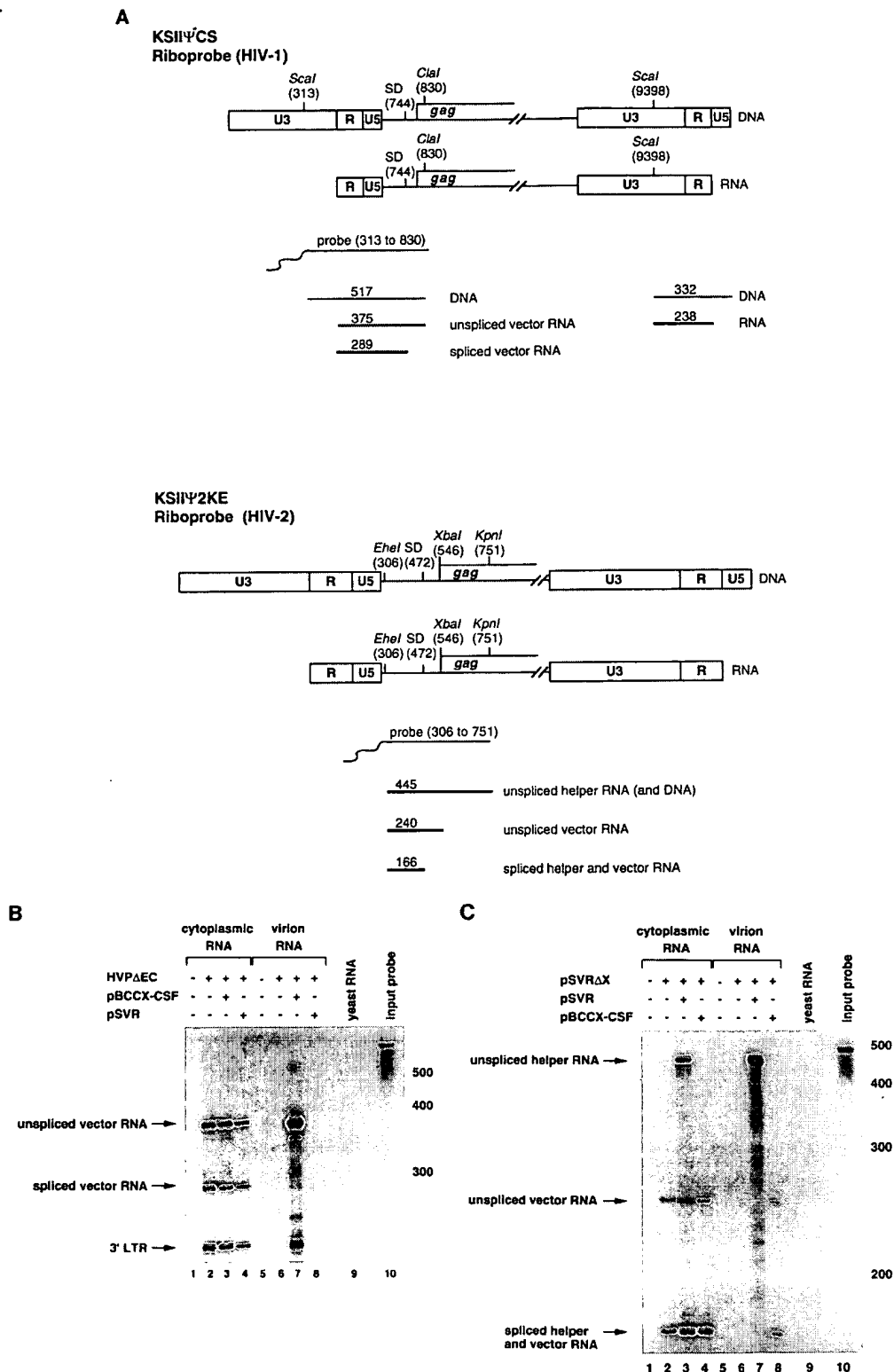


FIG. 2. Nonreciprocal RNA packaging by HIV-1 and HIV-2. HIV-1- and HIV-2-based vectors, HVPΔEC and pSVRΔX, were cotransfected into COS-1 cells with the HIV-1 or HIV-2 helper virus constructs pBCCX-CSF or pSVR, respectively, or alone. RNA isolated from the cytoplasm of the transfected cells or from virions was subjected to RNase protection analysis. (A) Predicted sizes of the protected fragments for the HIV-1- and HIV-2-specific riboprobes, KSIIΨCS and KSIIΨ2KE, respectively. SD, splice donor. (B) RNase protection analysis with the HIV-1-specific riboprobe, KSIIΨCS. Diagnostic bands for HIV-1 vector RNA are 375 nt (unspliced RNA), 289 nt (spliced vector RNA), and 238 nt (3' LTR). The relative levels of unspliced and spliced vector RNA are 1.9 in lane 3 and is 65.6 in lane 7. (C) RNase protection analysis with the HIV-2 specific riboprobe, KSIIΨ2KE. Diagnostic bands for unspliced HIV-2 helper virus and vector RNA are 445 nt, respectively, and a band of 166 nt is protected for spliced RNA from both constructs. The relative levels of unspliced and spliced helper RNA are 0.7 in lane 3 and 19.8 in lane 7. The relative levels of unspliced and spliced vector RNA are 0.4 in lane 4 and 0.4 in lane 8. Protection with control RNA (yeast RNA) and with the riboprobe without RNase treatment (input probe) is shown. The positions of RNA size markers are shown in nucleotides to the right of the panels B and C.

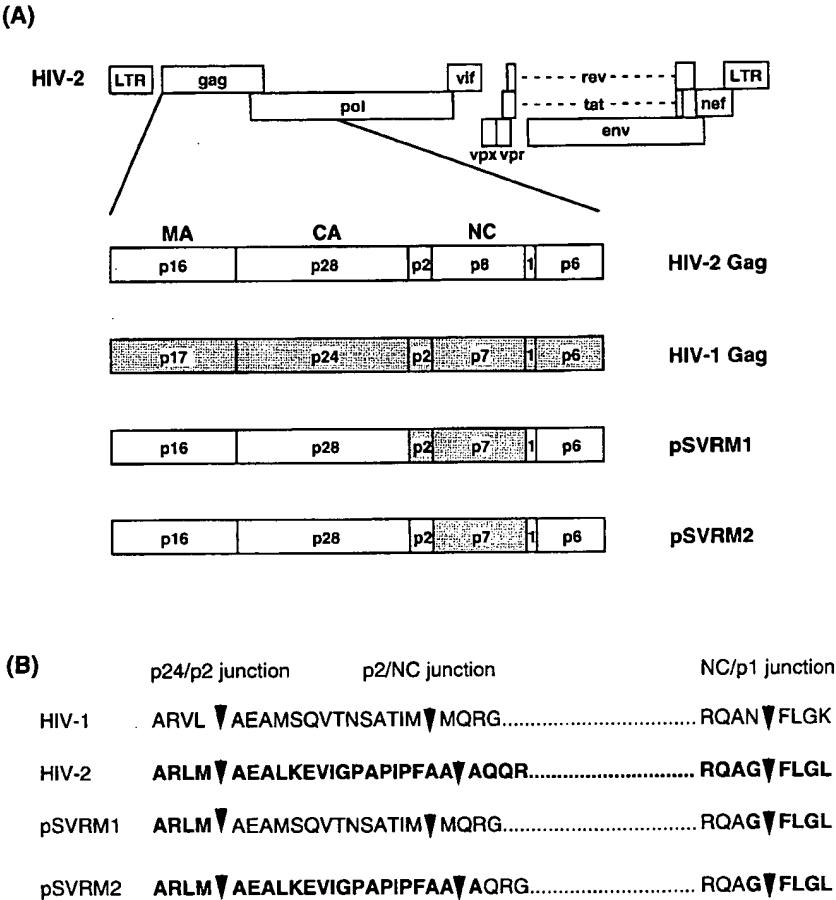


FIG. 3. Schematic representation of chimeric constructs used in this study. (A) Gag region of wild-type HIV-1 and HIV-2 and chimeric constructs, pSVRM1 and pSVRM2, showing the subdomains. HIV-1 sequences are shaded. MA, matrix; CA, capsid. (B) Junctions between the HIV-1 and HIV-2 sequences in the chimeric constructs. Amino acid residues derived from HIV-2 sequences are shown in boldface type. The dotted regions indicate NC residues. The predicted protease cleavage sites are indicated by arrowheads.

RNA (i.e., containing the HIV-2 5' untranslated region) in *cis* was examined and found to be similar to that of the parental HIV-2 construct (Fig. 6, compare lanes 7 and 8 to lane 6). Unspliced RNA was packaged preferentially compared to

spliced RNA. The NC domain of HIV-1, in the context of HIV-2 Gag polypeptide, is thus able to package both HIV-1 and HIV-2 RNAs.

DISCUSSION

Although similar in genomic organization, HIV-1 and HIV-2 are dissimilar in many ways. Previous studies on the transactivator proteins Tat and Rev of the two viruses have shown a lack of reciprocity (3, 5, 13, 15, 16, 18, 30, 32, 44). This is the first study to formally cross-complement *cis*- and *trans*-acting factors involved in packaging. HIV-1 has been reported to package HIV-2 RNA (2, 39) and simian immunodeficiency virus RNA (42); however, the ability of HIV-2 to package HIV-1 RNA was not investigated. In this report, we demonstrate that while HIV-1 is able to package both HIV-1 and HIV-2 RNA, HIV-2 is not able to package HIV-1 RNA. The HIV-1 helper virus packaged the HIV-2 vector RNA with a lack of preference for unspliced RNA over spliced RNA. In contrast, the HIV-1 p2 and NC domains, in the context of HIV-2 Gag polypeptide, showed a preference for unspliced RNA.

The HIV-2 helper virus did not package an HIV-1 vector, nor did it package an HIV-2-based vector. The failure of HIV-2 helper virus to package pSVRΔX was surprising since

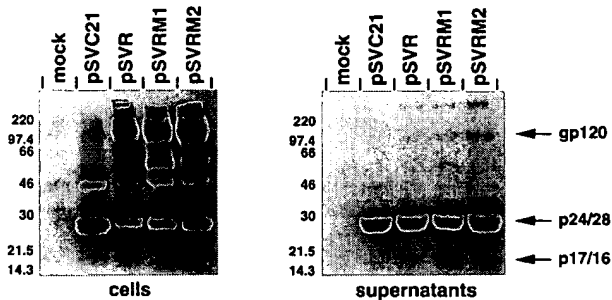


FIG. 4. Immunoprecipitation analysis of chimeric constructs. Wild-type proviral constructs pSVC21 (HIV-1) and pSVR (HIV-2) and chimeric constructs pSVRM1 and pSVRM2 were transfected into COS-1 cells. The cells were labelled with [³⁵S]methionine from 44 to 48 h after transfection, and cell and virion lysates were subjected to immunoprecipitation analysis with pooled human sera from a panel of HIV-2-infected individuals. The molecular mass markers are shown to the left in kilodaltons. The predicted positions of viral proteins gp120, p24/28, and p17/16 are indicated.

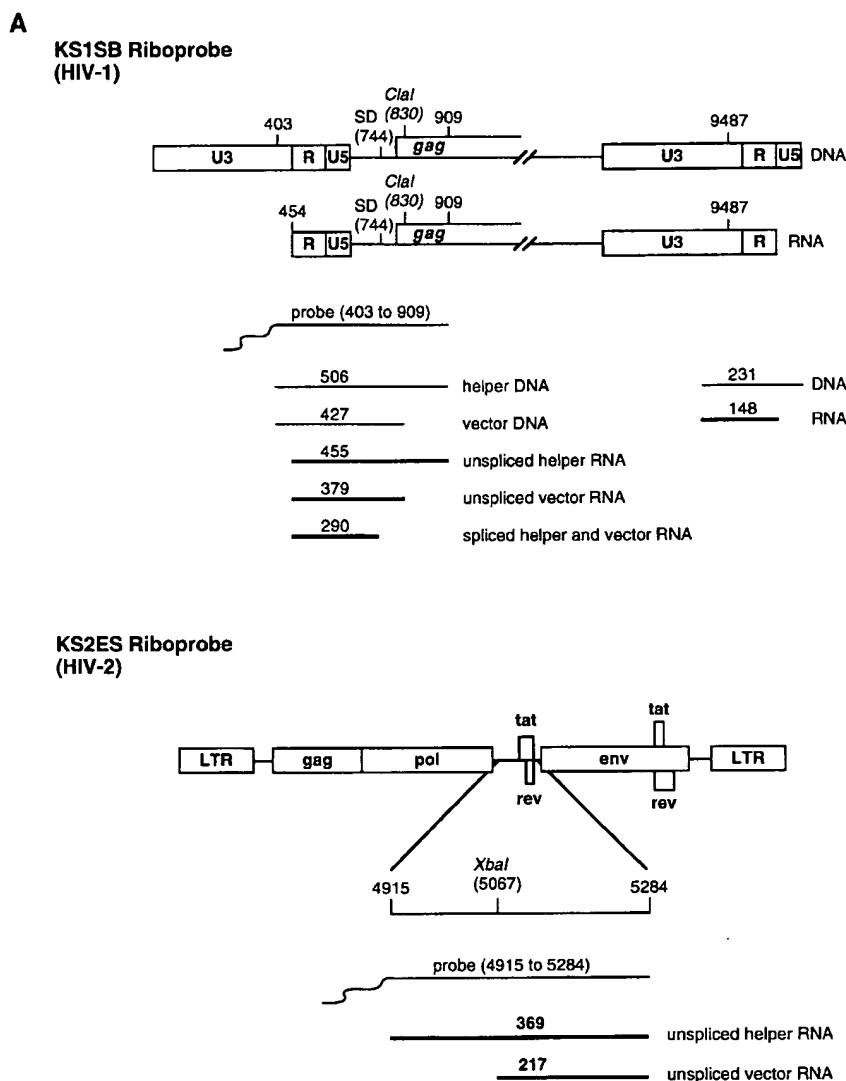


FIG. 5. RNase protection analysis of chimeric proviruses. (A) Predicted sizes of the protected fragments for the HIV-1- and HIV-2-specific riboprobes, KS1SB and KS2ES, respectively. SD, splice donor. (B) COS-1 cells were transfected with the HIV-1 vector HVPΔEC alone (lanes 2 and 8) or with pSVC21 (lanes 3 and 9), pSVR (lanes 4 and 10), pSVRM1 (lanes 5 and 11), or pSVRM2 (lanes 6 and 12) or mock transfected (lanes 1 and 7). Cytoplasmic RNA (lanes 1 to 6) and virion RNA (lanes 7 to 12) were subjected to RNase protection analysis with an HIV-1 specific riboprobe, KS1SB. The positions of unspliced helper virus RNA (455 nt), unspliced vector RNA (379 nt), and spliced helper and vector RNA (290 nt) are indicated by arrows. The relative levels of unspliced vector packaged by the various helper constructs compared to HIV-1 helper virus (given an arbitrary value of 1) are 0.31 in lane 11 and 0.03 in lane 12. Protection with yeast RNA (lane 13) and the riboprobe without RNase treatment (lane 14) is shown. The RNA molecular size markers (in nucleotides) are shown (lane 15). (C) COS-1 cells were transfected with the HIV-2 vector pSVRΔAX alone (lanes 2 and 7), or with pSVR (lanes 3 and 8), pSVC21 (lanes 4 and 9), or pSVRM1 (lanes 5 and 10) or mock transfected (lanes 1 and 6). Cytoplasmic RNA (lanes 1 to 5) and virion RNA (lanes 6 to 10) were subjected to RNase protection analysis with an HIV-2-specific riboprobe, KS2ES. The positions of unspliced HIV-2 helper virus RNA (369 nt) and unspliced vector RNA (217 nt) are indicated by arrows. The relative levels of unspliced vector and unspliced helper RNA are 3.2 in lane 3, 17.4 in lane 5, 0.9 in lane 8, and 1.1 in lane 10. Protection with yeast RNA (lane 11) and the riboprobe without RNase treatment (lane 12) are shown. The RNA molecular size markers (in nucleotides) are shown (lane 13).

the vector contained the entire 5' untranslated region of the HIV-2 genome. Arya and Gallo (2) showed that an HIV-2 construct in which *gag* and *pol* sequences had been deleted was packaged by HIV-1, but they did not test the ability of HIV-2 helper virus to package the HIV-2 vector, nor did they report on the relative levels of unspliced to spliced vector RNA that were packaged by HIV-1. Poeschla et al. reported the packaging of an HIV-2-based vector by an HIV-2 packaging cell line by measuring vector transduction efficiencies (39). Their HIV-2 vector had its *gag* and *pol* sequences deleted; however, some of the p17 (matrix) domain of Gag remained, but the

extent of the deletion was not documented. There are several possible explanations for the failure of HIV-2 helper virus to package pSVRΔX: first, there may be *cis*-acting sequences in the *gag* or *pol* genes, absent from pSVRΔX, that are required for RNA packaging; second, the packaging capacity of HIV-2 could be saturated by packaging of wild-type HIV-2 RNA; or third, RNA packaging in HIV-2 could be linked to translation of Gag. Previous studies of HIV-2-packaging signals have been restricted to the 5' untranslated region of the genome, where deletion of sequences upstream of the major splice donor caused a reduction in genomic RNA encapsidation (19, 35);

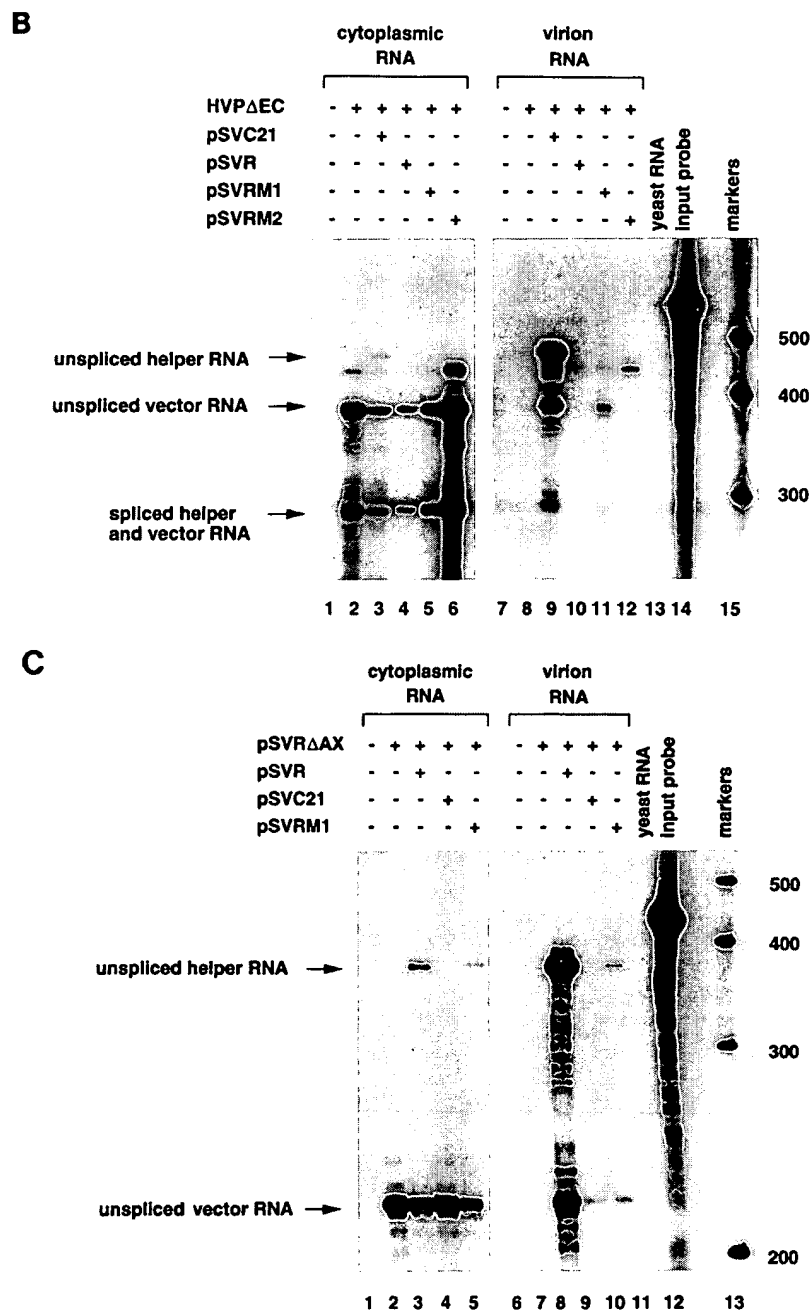


FIG. 5—Continued.

however, neither study addressed the question of which sequences are sufficient for encapsidation in HIV-2. The packaging capacity of HIV-2 was not saturated, since a second vector, pSVRΔAX, was packaged by HIV-2 helper virus. A link between packaging and translation in HIV-2 is possible, although for HIV-1 it has previously been shown by several groups that HIV-1-based vectors lacking *gag* and *pol* sequences can be efficiently packaged by HIV-1 (34, 37, 41). A second HIV-2-based vector, pSVRΔAX, which contained an additional 366 nt of *Gag*-coding sequences, was packaged by HIV-2

helper virus. The role of HIV-2 *gag* sequences in packaging of HIV-2-based vectors is currently being investigated.

The HIV-1 and HIV-2 NC proteins are very similar, with 60% identical amino acid residues and with conservative substitutions in much of the rest of the proteins. We attempted to identify the residues within NC that are responsible for the ability to recognize HIV-1 RNA. The chimeric proviruses we constructed expressed and processed viral proteins and released virus particles normally, indicating that the domain swaps had not affected protease mediated cleavage, virus as-

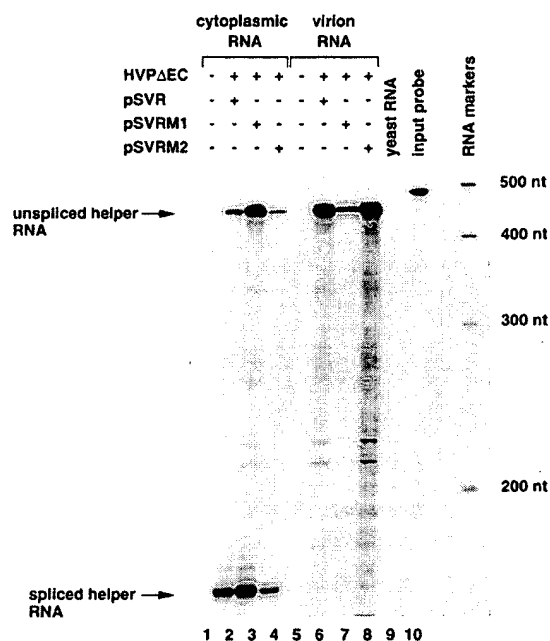


FIG. 6. RNA encapsidation in *cis* by chimeric proviruses. COS-1 cells were cotransfected with the HIV-1 vector HVPΔEC and pSVR (lanes 2 and 6), pSVRM1 (lanes 3 and 7), or pSVRM2 (lanes 4 and 8) or mock transfected (lanes 1 and 5). Cytoplasmic RNA (lanes 1 to 4) and viral RNA (lanes 5 to 8) was subjected to RNase protection analysis with an HIV-2-specific riboprobe, KSH1P2KE. The positions of unspliced HIV-2 helper virus RNA (445 nt) and spliced RNA (240 nt) are indicated by arrows. The relative levels of unspliced helper RNA and spliced RNA are 0.6 in lane 2, 1.0 in lane 3, 0.6 in lane 4, 47.0 in lane 6, 57.2 in lane 7, and 23.4 in lane 8. The relative levels of packaging of unspliced helper RNA compared to wild-type HIV-2 (given an arbitrary value of 1) are 0.26 for pSVRM1 and 1.57 for pSVRM2. Protection with yeast RNA (lane 9) and with the riboprobe without RNase treatment (lane 10) is shown. RNA size markers are shown to the right (sizes in nucleotides are indicated).

sembly, or budding. The ability of the chimeras to package an HIV-1 vector was assessed in comparison to wild-type HIV-1 and HIV-2 helper viruses. The presence of the HIV-1 p2 and NC domains conferred on the HIV-2 chimeric construct the ability to package an HIV-1 vector. The level of encapsidation of the HIV-1 vector was lower than that observed for wild-type HIV-1, suggesting that other domains of the HIV-1 Gag polypeptide are required for optimal encapsidation of HIV-1 RNA. A chimera in which only the NC domain had been exchanged also encapsidated the HIV-1 vector, although the level of packaging was reproducibly lower than that seen for the chimera containing both the p2 and NC domains of HIV-1, suggesting that there are residues within the HIV-1 p2 domain that contribute to the specific recognition of the HIV-1 packaging signal. In contrast to NC, the p2 domains of HIV-1 and HIV-2 are quite different, with only 35% amino acid homology. This study suggests a further important role for the HIV-1 p2 domain, which has previously been shown to be essential for virion assembly and viral infectivity (27, 38).

Recognition of the *cis*-acting packaging signals on the viral genomic RNA is carried out by the Gag polypeptide. Proteolytic cleavage of Gag polypeptide to its cleavage products occurs during or after budding of the progeny virions. Thus, although NC, with its nucleic acid binding capacity, probably constitutes the major high-affinity interaction involved in packaging, the discrimination of genomic RNA might be conferred by other regions within Gag either by directly interacting with the RNA-packaging signal or by influencing the three-dimen-

sional array of RNA binding motifs in NC such that they are optimized for binding the genome in its monomeric or dimeric state. The effect of p2 in this study was striking and reproducible, and further studies are under way to analyze its function in RNA encapsidation. Chimeras with groups of amino acid residues within HIV-2 NC exchanged for the corresponding residues from HIV-1 did not package HIV-1 RNA to a measurable level (data not shown). The low level of packaging of HIV-1 RNA by the chimera containing just the HIV-1 NC domain made it difficult to assess the role in viral RNA recognition and encapsidation of individual residues of HIV-1 NC. It may be necessary to use *in vitro* RNA-protein binding assays to address the question of which residues within HIV-1 Gag convert HIV-2 Gag into a polypeptide capable of packaging HIV-1 RNA.

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